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A T Cell Intrinsic Role of Id3 in a Mouse Model for Primary Sjögren's Syndrome

HongMei Li,[1,2] MeiFang Dai,[2] and Yuan Zhuang[2,*]
[1]Institute of Developmental Biology
and Molecular Medicine
Fudan University
Shanghai 200433
China
[2]Department of Immunology
Duke University Medical Center
Durham, North Carolina 27710

Summary

Sjögren's syndrome is an autoimmune disease with clinical hallmarks of keratoconjunctivitis sicca (dry eyes) and xerostomia (dry mouth). The genetic basis of this autoimmune disease is poorly understood. Id3 is an immediate early-response gene in growth regulation and is involved in TCR-mediated T cell selection during T cell development. Here, we show that Id3-deficient mice develop many disease symptoms found in primary Sjögren's syndrome patients including dry eyes and mouth, lymphocyte infiltration in lachrymal and salivary glands, and development of anti-Ro and anti-La antibodies. Adoptive transfer experiment indicated a T cell intrinsic role for Id3 in the development of Sjögren's symptoms. Furthermore, genetic ablation of T cells or neonatal 3 day thymectomy in Id3-deficient mice showed a rescue of disease symptoms, suggesting a thymic origin of autoimmune T cells. Thus, this study establishes a critical connection between Id3-mediated T cell development and autoimmune diseases.

Introduction

Sjögren's syndrome is an autoimmune disease in which immune cells chronically attack the lachrymal and salivary glands. Sjögren's syndrome is considered secondary if it occurs in the setting of another disease such as rheumatoid arthritis or systemic lupus erythematosis. Sjögren's syndrome is otherwise termed primary and may be associated with extraglandular features, including fatigue, arthritis, pulmonary involvement, interstitial nephritis, peripheral neuropathy, and vasculitis (Fox et al., 1984, 2000). Patients with primary Sjögren's syndrome also produce a diverse array of serum autoantibodies such as anti-Ro and anti-La, providing evidence of an autoimmune origin for this disease (Jonsson et al., 2002; St Clair, 1992).

It is generally difficult to determine the cause of human autoimmune disease because the clinical features often occur after the development of immunological abnormalities and tissue pathology. In the past two decades, the mouse has become a major model organism in the study of autoimmune diseases. Sjögren's syndrome has been extensively studied in nonobese diabetic (NOD) mice and MRL/lpr mice (van Blokland and Versnel, 2002). However, NOD mice were initially established as a model for insulin-dependent diabetes mellitus, and MRL/lpr mice were primarily used as a model for systemic lupus erythematosus (SLE). While both strains mimic some histological and serological manifestations of human Sjögren's syndrome, only NOD mice show loss of tear and saliva secretory function. The Sjögren's syndrome in MRL/lpr seems to be secondary of SLE, whereas the Sjögren's syndrome in NOD mice occurs independent of diabetes (Robinson et al., 1998b). In both animal models, the genetic background plays important roles in the development of Sjögren's syndrome. It is clear that multiple genetic loci contribute to the disease development in both NOD and MRL/lpr mice (Bolstad and Jonsson, 2002).

Id3 is a 13 kDa nuclear protein which is upregulated in a broad range of cell types upon stimulation with many growth and differentiation signals (Christy et al., 1991; Norton, 2000), including TCR- and BCR-mediated signals in the T and B lymphocytes, respectively (Bain et al., 2001; Pan et al., 1999). Id3 is a unique member of the basic-helix-loop-helix (bHLH) transcription factor family. Most bHLH proteins bind the CANNTG (E box) DNA motif as protein dimmers, with the basic region making contact with DNA and the HLH domain mediating protein dimerization. Id3 lacks the basic region needed for DNA binding but retains the functional dimerization domain. When bound to other bHLH proteins, Id3 prevents the resulting dimers from binding to DNA (Benezra et al., 1990). Biochemical and genetic studies demonstrate that Id3 primarily dimerizes with and consequently inhibits the function of E2A, a bHLH protein that is also broadly expressed and is essential for lymphocyte development (Kee et al., 2000).

Functions of Id3 in the immune system were supported by studies of Id3-deficient ($Id3^{-/-}$) mice. $Id3^{-/-}$ mice show reduced ability to mount humoral immune responses to several model antigens including DNP-keyhole limpet hemocyanin (KLH), a T-dependent antigen, and DNP-Ficoll, a type II T-independent antigen (Pan et al., 1999). These defects in humoral immunity were partially attributed to the involvement of Id3 in the BCR-mediated signaling pathway. A role for Id3 in T cell development has also been reported (Rivera et al., 2000). $Id3^{-/-}$ mice show a reduced percentage of single positive cells in the thymus. A thorough test of Id3 deficiency on various TCR transgenic backgrounds revealed impairments in both positive and negative selection. A more recent work further demonstrated a critical role for Id3 downstream of pre-TCR and TCR signals (Bain et al., 2001). Both pre-TCR and TCR signals lead to transcriptional activation of Id3, which antagonize E2A activities. These findings are in agreement with the observation that E2A-deficient mice exhibit enhanced positive and negative selection of single positive thymocytes. These complementary genetic data strongly indicate that the antagonistic interaction between Id3 and E2A plays an important role in TCR-mediated T cell selection during thymopoiesis, and genetic alteration

*Correspondence: yzhuang@duke.edu

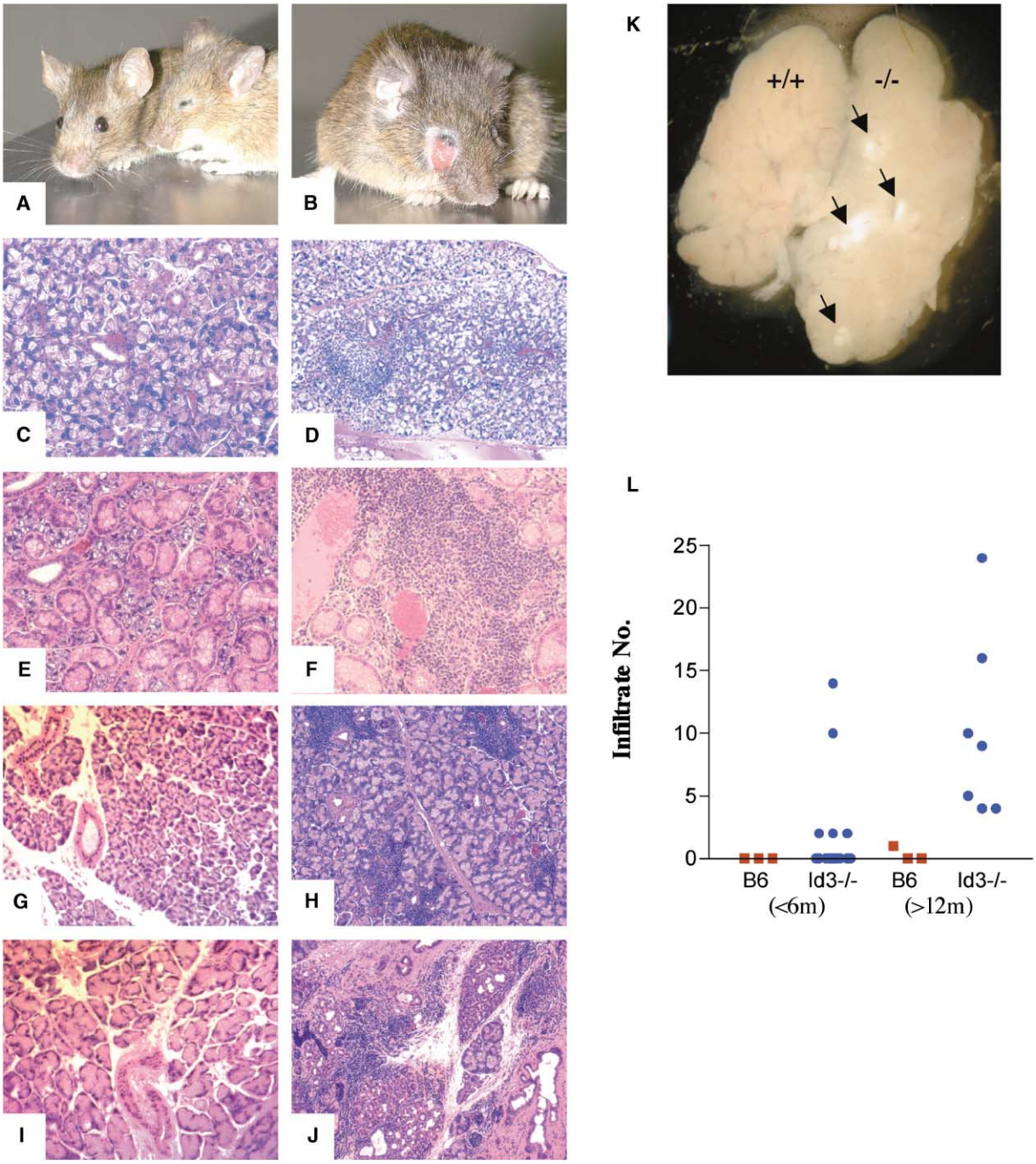

Figure 1. Pathological Evidence of Sjögren's Syndrome in Id3$^{-/-}$ Mice

(A) The eye problem is evident in a 7.5-month-old Id3$^{-/-}$ (right) mouse. Mouse on the left is an Id3$^{+/-}$ littermate control.

(B) A 16-month-old Id3$^{-/-}$ mouse develops skin lesions around the eyes.

(C–J) Haematoxylin and Eosin (H&E) staining of lachrymal and salivary glands. All pictures were taken with the original magnification of 10 × 20 lenses. (C–F) Extraorbital lachrymal glands (C and D) and submandibular salivary glands (E and F) from 6-month-old wild-type (C and E) and Id3$^{-/-}$ mice (D and F). (G–J) Parotid salivary glands (G and H) and extraorbital lachrymal glands (I and J) from 16.5-month-old wild-type (G and I) and Id3$^{-/-}$ (H and J) mice.

(K) A representative photo of freshly isolated extraorbital lachrymal glands from wild-type and Id3$^{-/-}$ mice. White spots are visible in the Id3$^{-/-}$ tissue (indicated by arrows), but not in the wild-type.

(L) Counting of lymphocyte infiltrates present in lachrymal and salivary glands from Id3$^{-/-}$ and wild-type mice. Each dot represents the infiltrate score from one mouse. Mice are divided into two age groups, either younger than 6 months or older than 12 months.

of this pathway may affect T cell-mediated immunity. Indeed, a recent study of E2A conditional knockout mice revealed an age-dependent autoimmunity due to loss of E2A in the T cell lineage (Pan et al., 2004).

Despite the relatively mild defects in T cell development and impairment in humoral immunity, Id3 knockout mice are fully viable and fertile. Given the age-dependent defects observed in E2A conditional knockout mice, it

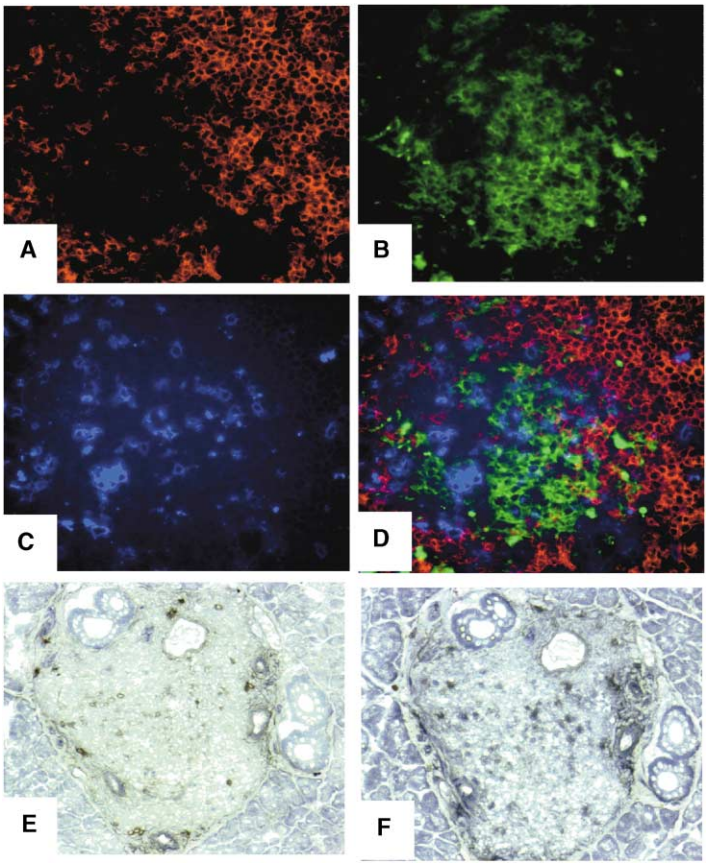

Figure 2. Identification of Cell Types Present in the Infiltrates of Aged Id3$^{-/-}$ Mice

(A–D) Immunofluorescent microscopic analysis of lymphocyte infiltrates in extralachrymal gland from a 1.5-year-old Id3$^{-/-}$ mouse. Frozen sections were stained with fluorescent labeled B220 (red, [A]) as a B cell marker, CD4 (green, [B]), and CD8 (blue, [C]) as T cell markers. Staining for B220 and CD4 was performed on the same section, and staining for CD8 was performed on the adjacent section. Images were superimposed (D) to show relative locations of individual cell populations. All images were taken with 40 × 10 original magnifications. Lymph node and nondiseased tissue were used as positive and negative controls, respectively (not shown).

(E and F) Immunohistochemistry analysis of frozen section of a parotid gland from a 1.5-year-old Id3$^{-/-}$ mouse. INF-γ (E) and IL-4-expressing (F) cells were labeled with brown color. Haematoxilin (light blue) was used as a counter stain. The large nodule structure in the middle is a lymphocyte infiltrate that is surrounded by gland and ductal tissues. Most nuclei were washed off from the frozen section by the end of staining process.

remains to be determined whether the early defect in T cell selection has any impact on T cell-mediated immunity throughout the normal life span of Id3-deficient mice. Here, our study reveals a multistage progression to human-like primary Sjögren's syndrome in Id3$^{-/-}$ mice. Significantly, our data establish a critical connection between Id3 function in thymopoiesis and the development of this unique autoimmune disease.

Results

Lymphocyte Infiltration in Lachrymal and Salivary Glands

During breeding of Id3$^{-/-}$ mice, we find that both males and females tend to have difficulties in maintaining fully opened eyelids beginning around six months of age (Figure 1A). Approximately 10% of Id3$^{-/-}$ mice show this phenotype between 6–10 months of age (13 out of 102 Id3$^{-/-}$ mice recorded). Some of these mice eventually develop skin lesions around the eyes after one year of age due to excessive scratching of the affected eyes (Figure 1B). The number of Id3$^{-/-}$ mice showing phenotypic abnormalities in the eyes increases to more than 80% after 1 year of age (35 out of 41 Id3$^{-/-}$ recorded). Histological and electron microscopy examination of the eyeballs and surrounding tissues did not reveal any bacterial or viral infections or anatomical abnormalities except for lymphocyte infiltration in the lachrymal glands and salivary glands (Figures 1C–1J), which are exocrine tissues responsible for tear and saliva secretion, respectively. Lymphocyte infiltration in lachrymal and salivary glands of Id3$^{-/-}$ mice is observed as early as 2 months of age and becomes more frequent at around 6 months of age (Figures 1D and 1F) even without visible abnormalities in the eyes. Many infiltrating lymphocytes are found in perivascular or periductal areas and have focal appearance (e.g., Figure 1H). Tissue destruction in lachrymal gland is also observed in aged Id3$^{-/-}$ mice (Figure 1J). These histopathology features are absent in age matched wild-type mice (Figures 1C, 1E, 1G, and 1I). In addition to the histopathological abnormalities, lachrymal glands from aged Id3$^{-/-}$ mice often contain opaque spots (Figure 1K), which correlate with the tissue damage observed on tissue sections. To further evaluate the age-dependent appearance of histopathology, we quantified numbers of lymphocyte infiltrates found in lachrymal and salivary glands from different age groups (Figure 1L). While infiltrating lymphocytes can be found in younger Id3$^{-/-}$ mice, the severity of infiltrates increased dramatically with age. The wild-type controls did not show significant numbers of infiltrates in either young or aged groups.

Lymphocytes Present in the Infiltrates

To further examine the cell types present in the infiltrates, we performed immunofluorescent microscopic analysis on affected gland tissues from aged Id3$^{-/-}$ mice. Lineage-specific markers were used to show the presence of CD4 and CD8 T cells and B cells (Figures 2A–2D). Both interferon-γ- and interleukin-4-secreting cells are detected within the infiltrates (Figures 2E and 2F). These observations confirm a lymphocyte-medi-

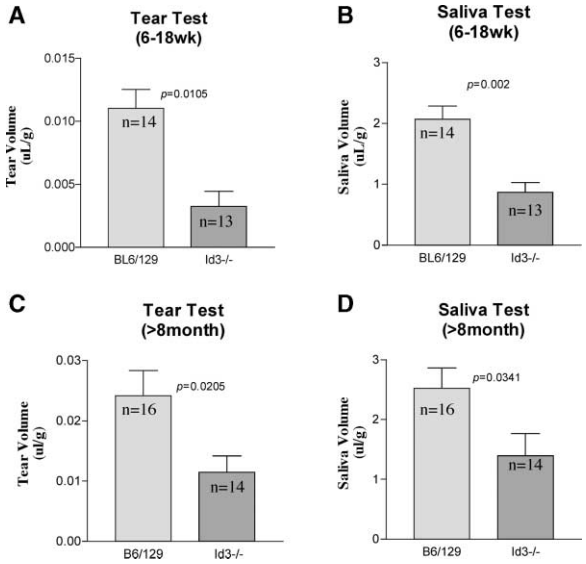

Figure 3. Tear and Saliva Secretion Tests of Young and Old Mice

Mice used in the tests were divided into young (6–18 weeks, [A] and [B]) and old (more than 8 months, [C] and [D]). The volume of tear and saliva is determined after pilocarpine stimulation. Values are normalized by body weight. Data are presented in bar graphs to show the mean value with standard error (SEM) for each genotype group. Numbers of individual animals used for each genotype group are indicated in the bar graph. Significant differences in tear and saliva flow volume are observed between Id3$^{-/-}$ and wild-type control mice for both age groups. P values for Student's t test are indicated between the paired groups.

ated chronic inflammation at the site. Flow cytometry analysis of both peripheral lymphocytes and tissue lymphocytes isolated from the salivary glands did not reveal any signs of significant imbalance of B or T cell subsets in aged Id3$^{-/-}$ mice (data not shown). The absence of overt abnormalities in peripheral lymphocytes is consistent with the fact that pathological abnormalities are relatively restricted to lachrymal and salivary glands (see Discussion).

Reduced Tear and Saliva Secretion

The phenotypic abnormalities in the eyes of Id3$^{-/-}$ mice and the detection of lymphocyte infiltrates in their lachrymal and salivary glands suggest that these animals may have developed a disease similar to Sjögren's syndrome, a systemic autoimmune disease found in humans. In humans, Sjögren's syndrome is characterized clinically by persistent dry eyes and dry mouth. In mice, the ability of exocrine tissues to secrete tear and saliva can be quantified after pilocarpine stimulation (Robinson et al., 1998a). We modified this method for easier and more accurate quantification (see Experimental Procedures) and used the modified method to compare tear and saliva flow volume in Id3$^{-/-}$ and control mice (Figure 3). We find that Id3$^{-/-}$ mice produce significantly less tear and saliva than wild-type controls. There is no significant difference in tear and saliva production between males and females of Id3$^{-/-}$ mice (data not shown). Significantly, this secretory defect occurs as early as 2–4 months of age before any other visible symptoms appear and persists throughout life. We also performed a similar test after 11 generation backcrossing of the Id3 mutation to the C57BL/6 background. Again, impairments in tear and saliva production in Id3$^{-/-}$ mice can be detected as earlier as 2 months of age (data not shown).

Detection of Autoantibodies in Id3$^{-/-}$ Mice

Human patients with Sjögren's syndrome often develop anti-Ro(SSA) and anti-La(SSB) autoantibodies. Low frequencies of anti-SSA and anti-SSB have been observed in NOD and MRL/lpr mice, two previously established animal models for Sjögren's syndrome (Skarstein et al., 1995; St Clair et al., 1990; Wahren et al., 1994). Our previous studies have demonstrated relatively normal levels of serum immunoglobulin in young adult Id3$^{-/-}$ mice (Pan et al., 1999). To further investigate the relevance of Id3$^{-/-}$ mice to human Sjögren's syndrome, we performed an ELISA test for anti-SSA and anti-SSB in Id3$^{-/-}$ mice. Both anti-SSA and anti-SSB antibodies can be detected at a significantly high frequency (six out of nine) in Id3$^{-/-}$ mice after 1 year of age, but not before (Figure 4). There is a strong correlation in antibody titers between these two autoantibodies among affected individuals (Figure 4C). A separate approach was also conducted to evaluate the autoreactivity of autoantibodies present in aged Id3$^{-/-}$ mice. Serum from Id3$^{-/-}$ mice was used in immunofluorescent microscopy analysis of lachrymal gland tissue section. We find that serum from aged Id3$^{-/-}$ mice displaying high titer of anti-SSA and anti-SSB also show autoreactivities to ductal tissues of lachrymal glands (Figure 4D). No significant autoreactive signals were detected in serum from aged wild-type mice (Figure 4D) or 6-month-old Id3$^{-/-}$ mice (data not shown). Similar staining pattern of autoantibodies has been reported in NOD mice (Goillot et al., 1991), although the exact autoantigens responsible for the staining pattern are not known in both NOD mice and our Id3$^{-/-}$ mice. Together, these results suggest that Id3$^{-/-}$ mice develop autoantibodies characteristic to those found in Sjögren's patients and NOD mice.

Symptoms of Sjögren's Syndrome Can Be Induced by Adoptive Transfer of Id3$^{-/-}$ Bone Marrow Cells

Id3 is known to be expressed in many tissue types including both hematopoietic systems and many solid tissues. To delineate the cause of the autoimmune diseases in Id3$^{-/-}$ mice, we performed a bone marrow adoptive-transfer test. Donor bone marrows are from Id3$^{-/-}$ mice that have been backcrossed to Bl/6 background for 11 generations. To avoid age-related variations, we used 2- to 4-month-old mice for both donors and recipients in this test. Tear and saliva tests were performed 2 months after adoptive transfer. We found that mice receiving Id3$^{-/-}$ bone marrow cells produced significantly less amount of tear and saliva after pilocarpine stimulation (Figure 5A). To further examine the age-dependent development of the disease, we saved five mice from each genotype group and waited for 10 months after the adoptive transfer before sacrificing the mice for additional tests. Flow cytometry analysis with donor marker (CD45.2) showed over 90% donor contribution of total lymphocytes population in the lymph nodes (Figure 5B). Significant numbers of infiltrate foci

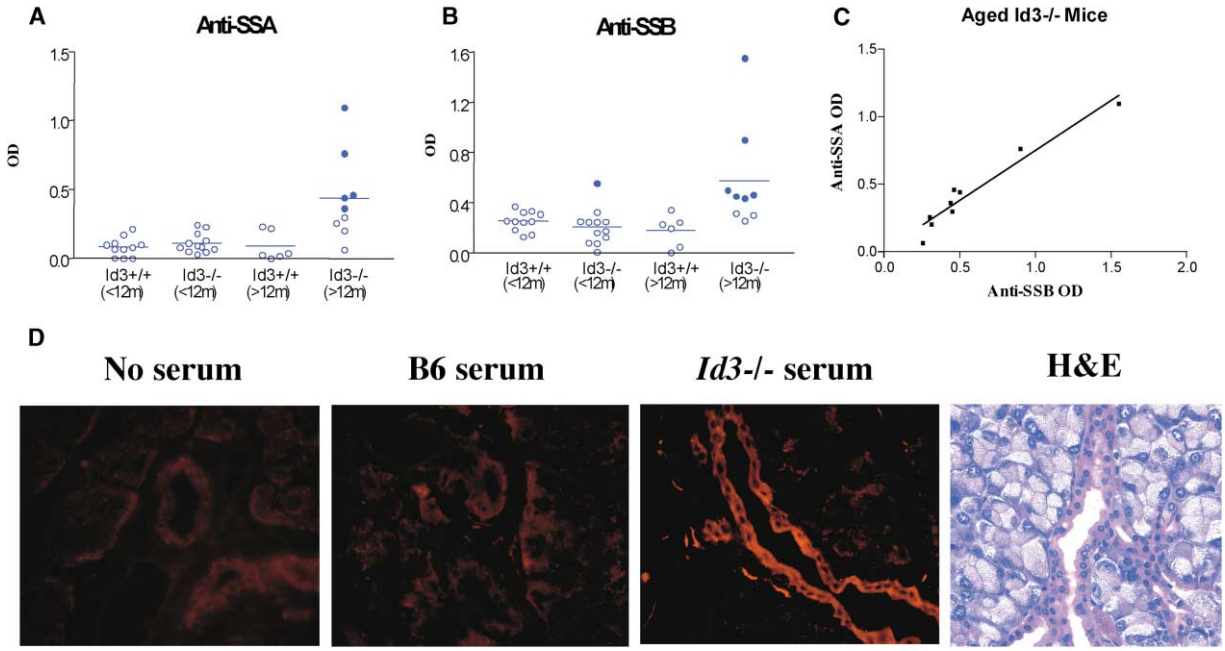

Figure 4. Detection of Autoantibodies in Aged Id3$^{-/-}$ Mice

(A and B) ELISA analysis of mouse serum for anti-Ro/SSA (A) and anti-La/SSB (B). Each circle represents one mouse. Lines indicate mean values of the experimental groups. OD values greater than two times of the mean values of the control group are considered positive (solid circle).

(C) The ELISA results of anti-SSA and anti-SSB for each individual among the aged Id3$^{-/-}$ group are displayed in the 2D plot. Significant correlation between OD values of anti-SSA and anti-SSB is observed ($p < 0.0001$).

(D) Immunofluorescent microscopy detection of autoantibodies against lachrymal gland tissues. Sera from a 12-month-old Id3 mouse and a 13-month-old wild-type mouse were used to probe lachrymal gland tissue sections prepared from a 3-month-old wild-type mouse. Secondary antibodies are biotin conjugated antimouse IgG (M.O.M. reagent from Vector Lab). Weak signals from B6 serum are due to background from using the secondary antibodies, which are also visible in the no serum control (far left panel). H&E staining of lachrymal gland tissue illustrates that the autoantibody staining is primarily on the ductal tissues.

were detected in the lachrymal and salivary glands of mice received Id3$^{-/-}$ bone marrow cells (Figure 5C). Although small numbers of infiltrate foci were also detected in mice received wild-type bone marrow cells, these foci were generally smaller in size compared with those found in Id3$^{-/-}$ bone marrow-derived mice (Figure 5D). In addition, opaque spots and extensive tissue damage were also observed in the lachrymal gland from Id3$^{-/-}$ bone marrow-derived mice, but not from the wild-type bone marrow-derived mice (data not shown). Together, these experiments showed that the disease symptoms observed in Id3$^{-/-}$ mice are due to Id3 deficiency within the hematopoietic lineage.

Id3$^{-/-}$ T Cells Play a Dominant Role

Previous studies have implicated that Id3 plays important roles in the development and functions of both T and B cells (Engel and Murre, 2001). To further investigate the role of individual lymphocyte populations in regulating tear and saliva production in Id3$^{-/-}$ mice, we next used purified lymphocytes in adoptive transfer experiments. Wild-type hosts were treated by sublethal dose of γ irradiation before the adoptive transfer. At 2 weeks after the adoptive transfer, tear and saliva secretion test showed that mice who received Id3$^{-/-}$ lymphocytes or purified T cells produced significantly less tears and saliva than those who received wild-type cells (Figures 6A–6D). In contrast, mice that received Id3$^{-/-}$ B cells did not show any significant deviation from the wild-type donors (Figures 6E and 6F). A time course analysis of tear and saliva flow volume showed that the suppression of tear and saliva secretion is transient in this assay (Figures 6B, 6D, and 6F). In addition, the suppression of tear and saliva secretion is more severe and of greater duration when total Id3$^{-/-}$ lymphocytes are used as donors in comparison with Id3$^{-/-}$ T cells alone, suggesting a cooperative role for Id3$^{-/-}$ B cells in suppressing exocrine gland function.

Id3$^{-/-}$ T Cells Play an Essential Role

We next tested whether T cells are essential for disease development in Id3$^{-/-}$ mice. The Id3$^{-/-}$ mice were crossed with the LAT-deficient mice, which are T cell deficient due to a developmental block in early stage of thymocyte development (Zhang et al., 1999). Id3 and LAT double deficient mice were analyzed for tear and saliva secretion. We found that elimination of T cells in Id3$^{-/-}$ mice corrects the defect in tear and saliva production (Figures 7A and 7B). Furthermore, histological analysis of 10-month-old Id3$^{-/-}$ LAT$^{-/-}$ mice did not detect any significant lymphocyte infiltration in their lachrymal and salivary glands (data not shown). In a parallel test, we crossed Id3$^{-/-}$ mice with the μMT mice, which are B cell deficient due to a mutation in the immunoglobulin heavy chain transmembrane domain (Kitamura and Rajewsky, 1992). Tear and saliva flow tests

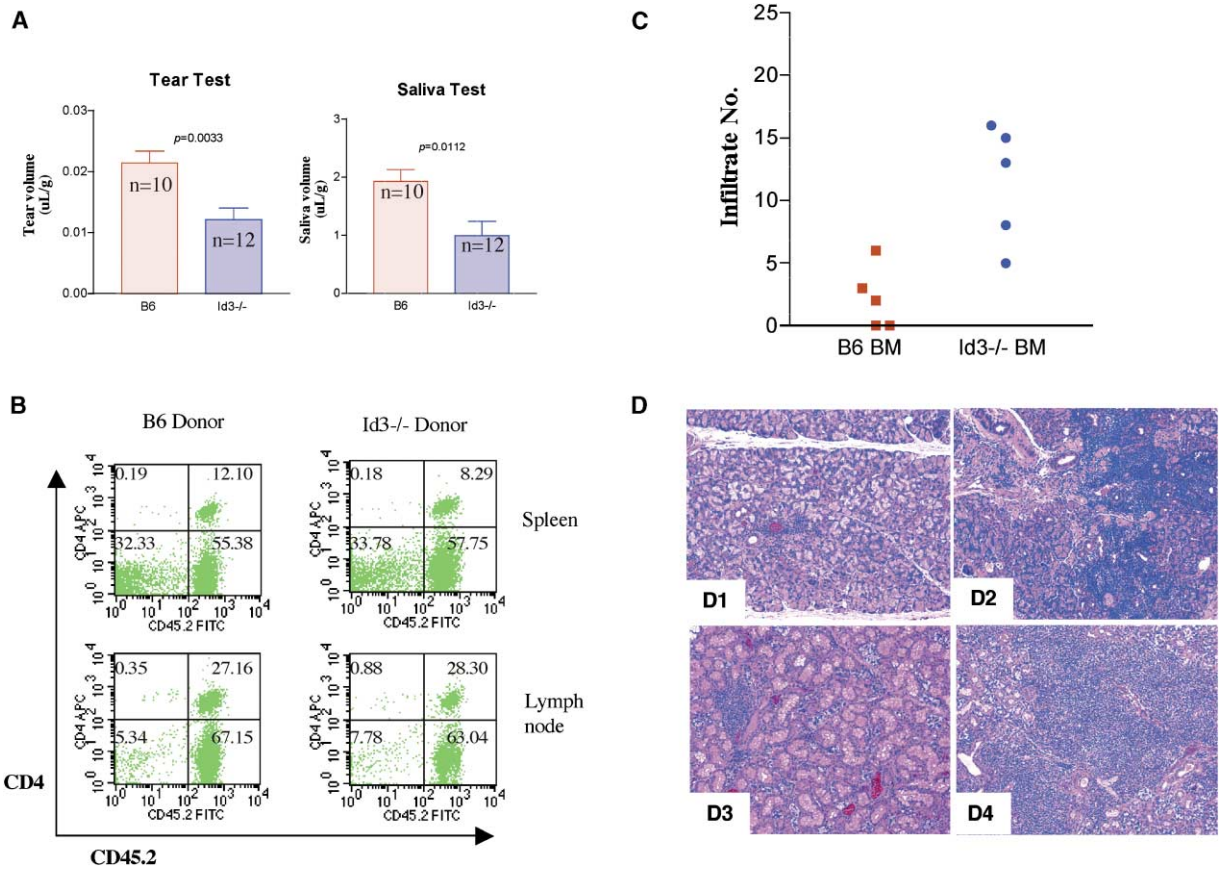

Figure 5. Bone Marrow Adoptive Transfer Recapitulates Id3 Knockout-Induced Disease Symptoms

(A) Tear and saliva tests were performed 2 months after adoptive transfer of bone marrow cells from either Id3$^{-/-}$ or Bl/6 mice to lethally irradiated wild-type hosts.

(B) Donor engraftment was evaluated by using CD45.2 as a donor-specific allelic marker in flow cytometry analysis. Representative data of CD4 and CD45.2 double staining on spleen and lymph node lymphocytes were shown.

(C) Histological score of infiltrates found in lachrymal and salivary glands of test animals 10 months after receiving either wild-type or Id3$^{-/-}$ bone marrow cells. Significant difference was found between two genotype groups ($p = 0.0049$).

(D1–D4) Representative slides of H&E staining used in (C). (D1) Extraorbital lachrymal gland after Bl6 bone marrow cell transfer. (D2) Extraorbital lachrymal gland after Id3$^{-/-}$ bone marrow cell transfer. (D3) Submandibula gland after Bl6 bone marrow cell transfer. (D4) Submandibula gland after Id3$^{-/-}$ bone marrow cell transfer.

show a significant difference between Id3$^{+/-}$ and Id3$^{-/-}$ genotype groups on the μMT homozygous background (Figure 7C), suggesting that defects in tear and saliva production at these age is independent of B cells. Together, this result indicates that T cells play a unique role in the development of autoimmune symptoms in Id3$^{-/-}$ mice.

Tear and Salivary Secretory Defects Can Be Rescued by 3 Day Thymectomy

To further address the origin of autoreactive T cells, we analyzed Id3$^{-/-}$ mice after neonatal thymectomy. Thymectomy was performed on 3-day-old neonates from a breeding between Id3$^{-/-}$ and Id3$^{+/-}$ mice. This procedure effectively eliminates sustained thymopoiesis but allows the survival and peripheral expansion of T cells generated prior to 3 day thymectomy. Indeed, flow cytometry analysis of these thymectomized mice confirmed the presence of significant amount of CD4 and CD8 T cells (Figure 7D). We tested the ability of these mice to secrete tear and saliva after pilocapine stimulation and found no significant defect associated with the Id3$^{-/-}$ mice when compared with Id3$^{+/-}$ mice (Figure 7E). This result suggests that the defect of tear and saliva secretion observed in Id3$^{-/-}$ mice requires a sustained production of T cells from thymus.

Discussion

The study presented here provided several lines of evidence to demonstrate that Id3-deficient mice develop autoimmune symptoms similar to human Sjögren's syndrome. First, these mice showed reduced ability to secrete tear and saliva and also showed the development of skin lesions caused by excess eye scratching at an older age. Second, both lachrymal and salivary glands of Id3$^{-/-}$ mice show lymphocyte infiltration before 6 months of age. A more severe and frequent histopathology in these gland tissues is observed after 1 year of age. Third, autoantibodies such as anti-SSA and anti-

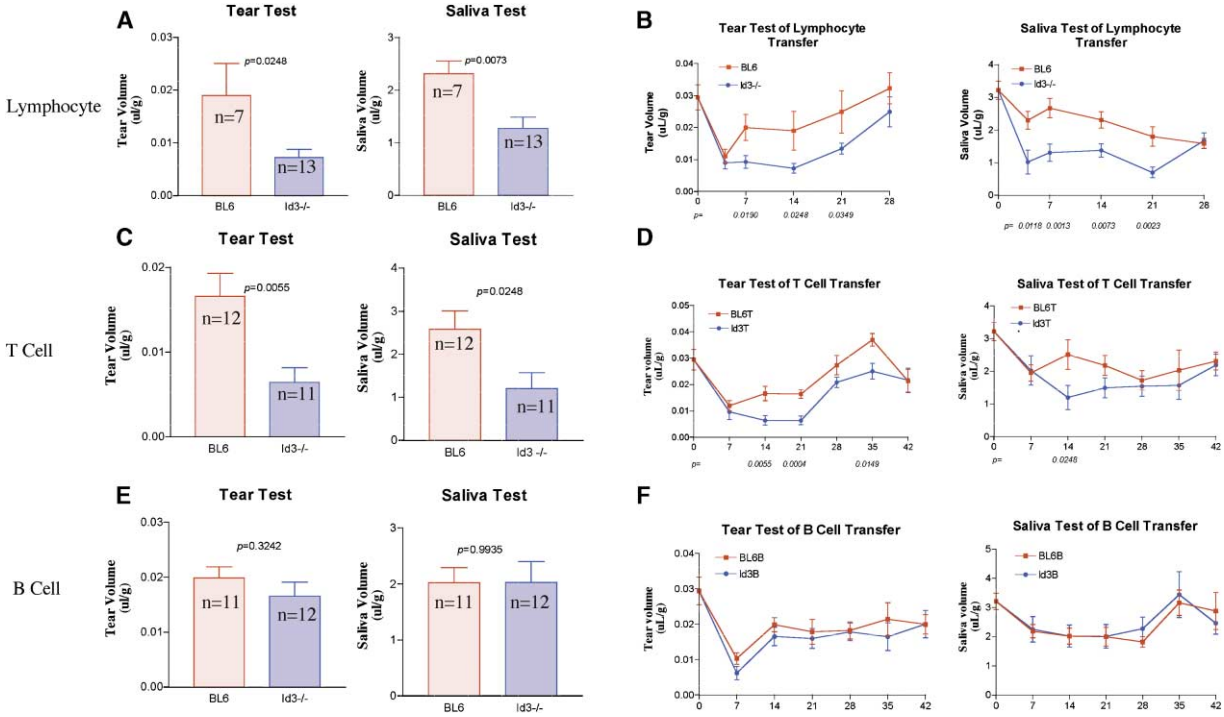

Figure 6. Tear and Saliva Secretion Test after Lymphocyte Adoptive Transfer

(A and B) Purified lymphocytes were transferred into sublethally irradiated wild-type hosts. Tear and saliva secretion tests were performed at weekly intervals. Results from the 2 week time point (A) and the entire time course (B) were shown in bar graphs and line graphs, respectively. Numbers of mice used in each experiment are indicated in the bar graph. At each time point, the difference between the Id3$^{-/-}$ and wild-type donors was evaluated by Student's t test. P values less than 0.05 are shown in the relevant time points.

(C and D) Same test as in (A) and (B) except purified T cells were used as donors.

(E and F) Purified B cells were used as donors.

SSB are detected in Id3$^{-/-}$ mice after 1 year of age. Given that the average life span of C57Bl/6 mouse is approximately 2 years in normal housing conditions, the age of autoantibody appearance in Id3$^{-/-}$ mice is equivalent to the middle age in humans when Sjögren's syndrome is most frequently detected. Fourth, we demonstrate that lymphocytes, in particular T lymphoyctes, play a dominant and essential role in the development of the disease. All these phenomena are in agreement with the criteria used in diagnosis of human Sjögren's syndrome (Jonsson et al., 2002; St Clair, 1992). Thus, we propose that Id3$^{-/-}$ mice may serve as a new animal model for human Sjögren's syndrome.

The nonobese diabetic (NOD) mice and MRL/lpr mice are two extensively studied animal models for Sjögren's syndrome (van Blokland and Versnel, 2002). NOD mice also develop insulin-dependent diabetes mellitus, and MRL/lpr mice develop systemic lupus erythematosus. We further evaluated Id3$^{-/-}$ mice for other possible autoimmune symptoms. First, we found that blood glucose is normal in the fasting state of Id3$^{-/-}$ mice, excluding diabetes. Second, urine protein level was not significantly altered in the test of a large group of Id3$^{-/-}$ mice, indicating proper kidney function. Third, histology studies of the pancreas, thyroid, kidney, liver, and lung did not reveal any gross damage, although lymphocyte infiltrates were occasionally detected in the kidney and lung of aged Id3$^{-/-}$ mice. The finding of lymphocyte infiltration in some, but not all, aged mice is consistent with the systemic nature of primary Sjögren's syndrome (Fox et al., 1984). The lack of other common autoimmune symptoms suggests that Id3$^{-/-}$ mice may serve as a unique animal model for primary Sjögren's syndrome.

Both adoptive transfer and genetic tests suggested that Id3$^{-/-}$ B cells alone are not sufficient to suppress tear and saliva secretory function at young age. This is in contrast to the observed autoantibody production in aged Id3$^{-/-}$ mice. These observations are not necessarily incompatible because humoral autoimmunity is often regulated by autoreactive T cells. In the absence of autoreative T cells, B cells alone may not be sufficient to initiate the autoimmune diseases. This type of T-B interaction has been shown in other autoimmune disease models. For example, it has been shown that rheumatoid arthritis can occur in NOD mice carrying a certain TCR trangene (Kouskoff et al., 1996). Although the TCR transgene plays essential roles in the disease development, the disease phenotype can be readily transferred by autoantibodies present in the diseased animals. Our study clearly showed that autoantibodies developed only in aged mice, and the timing of autoantibody production correlated with the appearance of exocrine tissue pathology and skin lesion (data not shown). It has been recognized in previous studies that Sjögren's syndrome is a chronic inflammatory disease that involves both T and B cells (Jonsson et al., 2002). In certain animal models, the disease phenotype can be transferred to mice by autoantibodies derived from either Sjögren's

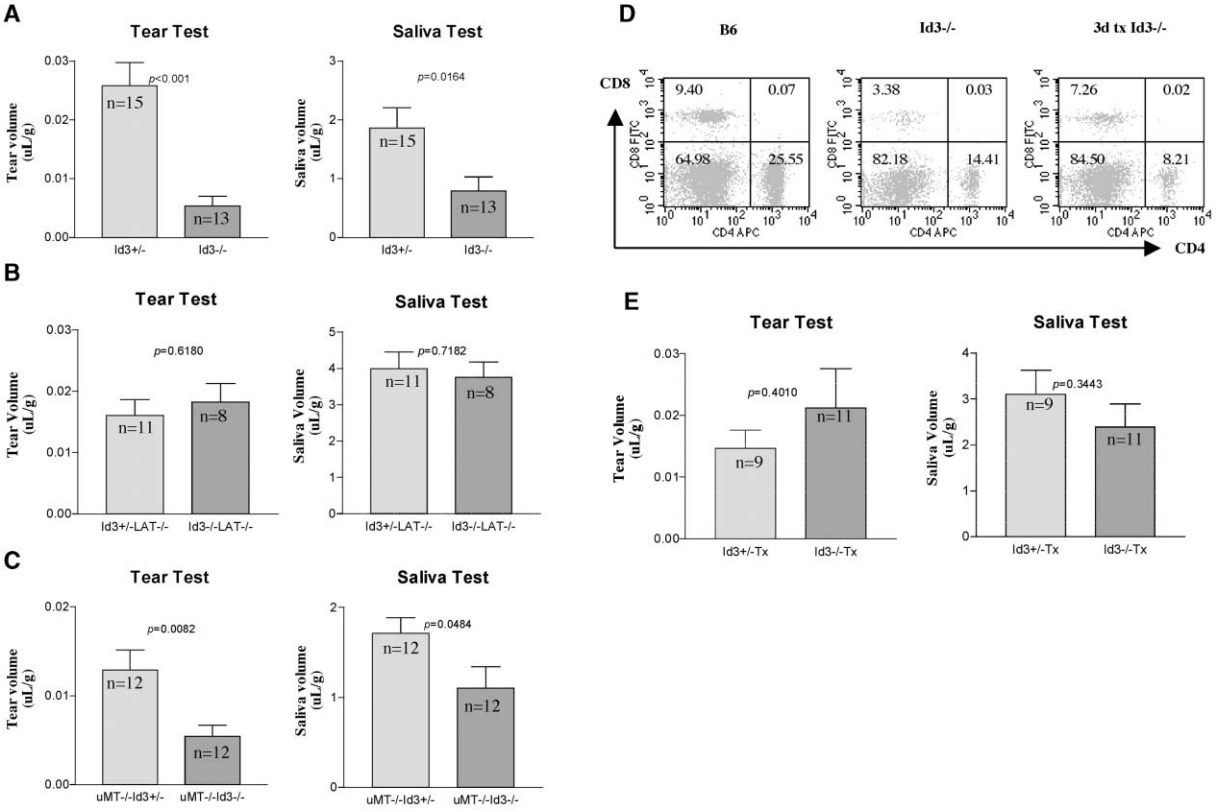

Figure 7. Tear and Saliva Secretion Tests of Id3$^{-/-}$ Mice on Various Genetic and Experimental Backgrounds

Bar graphs show the test result on C57Bl/6 background (A), LAT$^{-/-}$ background (B), or μMT homozygous background (C). In each graph, Id3$^{+/-}$ mice (on the left) were used as controls. Ages of mice used in this test were 28–34 weeks. Mean, SEM, sample size, and p values are shown in the bar graphs.

(D and E) The effect of 3 day thymectomy on Id3$^{-/-}$ mice. Thymectomy was performed on 3 day neonates born from mating between Id3$^{-/-}$ and Id3$^{+/-}$ mice. (D) Flow cytometry analysis of splenic T cells 1 year after thymectomy. The display of CD4 and CD8 staining is after gating on live (7AAD negative) lymphocytes. Age matched wild-type B6 mouse (far left) and Id3$^{-/-}$ mouse without thymectomy (middle) were included in the analysis as controls. (E) Tear and saliva secretion tests were performed at 2–4 months of age after 3 day thymectomy.

patients or diseased animals (Robinson et al., 1998a). One possible explanation of our observations is that autoreactive T cells may be involved in initiating the diseases at a young age, whereas autoreactive B cells may exacerbate the disease symptoms at older age. Further investigation of this idea may help establish a T cell-based early diagnosis of Sjögren's syndrome in humans.

A major issue in understanding organ-specific autoimmune disease is how and where autoimmune cells are developed. It has been shown that Id3 is a downstream effector molecule in the T cell receptor signaling pathway during positive and negative selection of thymocytes (Bain et al., 2001). Mice lacking Id3 show defects in both positive and negative selection. Therefore, it is possible that defects in thymopoiesis lead to the production of autoimmune T cells. It is also possible that the development of autoimmune T cells is independent of thymopoiesis. For example, Id3$^{-/-}$ T cells may have a subtle defect in TCR-mediated activation and proliferation. Thus, chronic stimulation by autoantigenes in the peripheral tissues may be solely responsible for the activation and expansion of autoreactive T cells. Several pieces of evidence argue against this idea. First, our previous studies showed Id3$^{-/-}$ T cells are indistinguishable from wild-type controls in regard to their ability to proliferate and produce cytokines after TCR stimulation (Pan et al., 1999). Second, the results from 3 day thymectomy test demonstrate the importance of sustained thymopoiesis on the induction of exocrinopathy. In these mice, Id3$^{-/-}$ T cells are readily detectable but no exocrine defects are observed. Extensive autoimmune defects have been reported in 3 day thymectomy experiments on autoimmune prone mouse strains such as BALB/c (Asano et al., 1996). The loss of regulatory T cells is thought to be the main reason for the observed autoimmunity. Our experiment was performed on Bl/6 strain background, which is considered to have relatively low incidence of developing autoimmune diseases (Roper et al., 2002). A general histopathology survey of 1-year-old mice received 3 day thymectomy detected lymphocyte infiltration in the lung and only sporadic infiltrates in salivary gland in both Id3$^{-/-}$ and Id3$^{+/-}$ mice (data not shown). This analysis confirms the effectiveness of thymectomy and further shows no significant pathological differences between Id3$^{-/-}$ and Id3$^{+/-}$ genotype groups. Thus, this study indicates that a sustained production of T cells from thymus is required for the devel-

opment of autoimmune symptoms characteristic of Sjögren's symptoms in Id3$^{-/-}$ mice. It supports the idea that defects in thymic T cell selection are responsible for the generation and accumulation of autoreactive T cells in Id3$^{-/-}$ mice.

In summary, our studies may provide a new animal model for primary Sjögren's syndrome. Although the role for Id3 in relevant human autoimmune disease has not been studied, our work provides one clue to the possible cause of autoimmune diseases. Future investigation of Id3 and Id3-mediated pathways should help us better understand how autoreactive T cells are developed in the thymus and subsequently influence the development of autoimmune diseases.

Experimental Procedures

Tear and Saliva Secretion Test

Mice were anesthetized with avertin. Pilocarpine Hydrochloride (Sigma) was dissolved in ddH_2O and injected intraperitoneally (0.5 μg/g body weight) to stimulate tear and saliva production. Tears were collected from both eyes by using a 5 μl sized microcapillary pipet at the 10 min and 12 min time point after pilocarpine injection. Saliva was collected with a 100 μl sized microcapillary pipet immediately after pilocarpine injection for a duration of 9 min. Tear and saliva secretion volumes are normalized by body weight. To avoid batch variations of pilocarpine and other system errors, tear and saliva tests were always performed against age-matched Bl/6 controls whenever appropriate.

Adoptive Transfer Test

Adoptive transfer tests were performed with young adult mice aged between 2–4 months. Recipients were either C57Bl/6 or C57Bl/6 CD45.1 congenic mice. Bone marrow transfer was carried out by introducing 6×10^6 total bone marrow cells into recipients one day after receiving 1100 rad γ irradiation. Tear and saliva tests were performed two month after adoptive transfer. The efficiency of donor engraftment was verified by FACS analysis of splenic and lymph node lymphocytes at the end of the experiments. Lymphocyte transfer was performed on mice receiving 200–300 rad γ irradiation. Total lymphocytes were purified from spleen and lymph nodes of donor mice by using lymphoprep (Nycomed Pharma) after lysing red blood cells. Subsequently, T cells were purified by using a T cell enrichment column (R&D Systems). B cells were stained with PE-conjugated B220 antibodies (BD Pharmingen) and then were purified on an AutoMac machine (Miltenyi Biotec) by using anti-PE-conjugated magnetic beads. $1–6 \times 10^6$ donor lymphocytes were delivered to each recipient. Tear and saliva tests were performed at weekly intervals posttransfer.

Histology, Immunofluorescent Microscopy, and Immunohistochemistry

Histology sections were prepared from paraffin-embedded tissues and stained by Haematoxylin and Eosin (H&E) method. We used the following criteria to score infiltrates for each mouse. Basically, one infiltrate is defined as 50 or more nucleated cells in the cluster. The score for each mouse is the total infiltrates present in histological sections prepared from one facial side including one extralachrymal gland, one parotid gland, and one submandibula gland. Immunofluorescent microscopic analysis was carried out with fluorescent conjugated antibodies on frozen tissue sections. Immunohistochemistry was performed with rat anti-mouse INF-γ or IL-4 antibodies (BD Pharmingen) on frozen sections. Biotin conjugated goat anti-rat IgG(H+L) antibody (Southern Biotechnology Associates, Inc.) was used in the subsequent step. Antibody binding to tissues was revealed by standard elite ABC peroxidase kit (Vector Laboratories) and DAB (brown color) as substrate (R&D Systems).

ELISA Test of Mouse Sera

Mouse SSA- or SSB-coated plates (Alpha Diagnostic, Inc.) were analyzed with 1:100 dilution of mouse serum. Both positive and negative controls were provided by the supplier. Experiment was done in duplicates. The optical density (OD) value for each serum test is derived after subtraction of blank and antibody controls. OD values greater than two times the mean values of the control genotype group are considered positive in this test.

3 Day Thymectomy

3 day thymectomy was performed as described (Bagavant et al., 2002). Basically, both thymic lobes were aspirated out with a glass pipet after making a midsternal incision. The wound was then closed with surgical glue before returning the pups back to the mother. The effectiveness of the surgical procedure was confirmed by visual examination of thymus at time of sacrifice.

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